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Cloning and characterization of cDNAS encoding carboxypeptidase-like proteins from the gut of Hessian fly larvae [*Mayetiola destructor* (Say)]☆

Xiang Liu[a], John P. Fellers[b], Yu Cheng Zhu[c], Navdeep S. Mutti[a], Mustapha El-Bouhssini[d], Ming-Shun Chen[a,b,*]

[a]*Department of Entomology, Kansas State University, Manhattan, KS 66506, USA*
[b]*Plant Science and Entomology Research Unit, USDA-ARS-GMPRC, 4008 Throckmorton Hall, Manhattan, KS 66506, USA*
[c]*USDA-ARS-JWDSRC, P.O. Box 346/141 Exp Stn Rd, Stoneville, MS 38776, USA*
[d]*International Center for Agricultural Research in the Dry Areas, Aleppo, Syria*

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Abstract

Transcriptomic analysis of the gut from Hessian fly larvae [*Mayetiola destructor* (Say)] identified nine cDNA clones that encode different carboxypeptidase-like proteins. Sequence comparison revealed that five of the nine cDNAs encoded very similar proteins with amino acid sequence identity over 96%. The other four cDNAs encoded diversified proteins with amino acid sequence identity less than 60%. Further sequence comparison with well characterized carboxypeptidases from other organisms revealed that these cDNAs encoded MDCP (*M. destructor* carboxypeptidase)-A1, MDCP-A2, MDCP-B, MDCP-BL, and MDCP-D. All residues characteristic of metallocarboxypeptidases including the HXXE motif were conserved among members. Northern blot analysis revealed various expression patterns for different gene groups in different developmental stages of *M. destructor*, suggesting that individual carboxypeptidases perform specific functions or have different specificities. Enzymatic activity assays demonstrated that both carboxypeptidases A and B are predominant in the larval stage, the only feeding stage of *M. destructor*, indicating a role in food digestion. The digestive role is further supported by the fact that 80% of the enzymatic activity in larvae occurred in the gut. Among these two types of enzymes, the activity of carboxypeptidase A was at least four times higher than that of carboxypeptidase B under the same conditions, suggesting that carboxypeptidase A is the major digestive enzyme in the gut of *M. destructor* larvae.

Keywords: Hessian fly; *Mayetiola destructor*; Carboxypeptidase; Gut; Digestive enzyme

1. Introduction

Since proteinaceous inhibitors were first found as defense molecules in plants (Green and Ryan, 1972), digestive enzymes have been targets for developing biopesticides to control herbivorous arthropod pests. Numerous digestive proteases and amylases have been characterized from many agriculturally important insects (Bown et al., 1998; Gaines et al., 1999; Cristofoletti et al., 2001; Coffeen and Wolpert, 2004; Gruden et al., 2004; Diaz-Mendoza et al., 2005; Zhu et al., 2005). Subsequently, various proteinaceous inhibitors have been identified against these enzymes from different sources (Farmer et al., 1992; Zhao et al., 1996; Koiwa et al., 2000; Moura and Ryan, 2001). In vitro assays with artificial diets have demonstrated that elevated levels of proteinaceous inhibitors may retard the growth, increase the mortality, and lower the fecundity of insects (Wolfson and Murdock, 1987; Franco et al., 2004). However, the detrimental effect of elevated levels of inhibitors can be shortly overcome by insects through changes in the composition of gut digestive proteases by compensating

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*Corresponding author. Tel.: +1 785 532 4719; fax: +1 785 532 6232.
E-mail address: mchen@ksu.edu (M.-S. Chen).

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for the loss of activity with induction of resistant proteases (Jongsma et al., 1995; Ishimoto and Chrispeels, 1996; Zhu-Salzman et al., 2003). Such adaptation enables insects to overcome plant defense mechanisms. Therefore, a better understanding of enzymatic composition and their expression profiling will improve strategies to control herbivorous arthropod pests.

Previous research on insect digestive proteases has been focused on endopeptidases that act on the initial phases of protein degradation, i.e. the cleavage of proteins into peptides. Nevertheless, most peptide products cleaved by endopeptidases cannot be absorbed by insect gut cells and need to be further hydrolyzed by exopeptidases and dipeptidases (Terra and Ferreira, 1994). Accordingly, a combination of inhibitors to endo-, exo-, and di-peptidases may be more effective and/or more durable than inhibitors to endopeptidases alone.

Mammalian carboxypeptidase, an exopeptidase, has been well characterized (Titani et al., 1975; Rees et al., 1983; Coll et al., 1991; Guasch et al., 1992; Aloy et al., 1998), but the studies of insect carboxypeptidases are limited to a few species including black fly *Simulium vittatum* (Ramos et al., 1993), corn earworm *Helicoverpa armingera* (Bown et al., 1998; Bown and Gatehouse, 2004), mosquitoes *Anopheles gambiae* and *Aedes aegypti* (Edwards et al., 1997, 2000), tsetse fly *Glossina morsitans* (Yan et al., 2002), bertha armyworm *Mamestra configurata* (Hegedus et al., 2003), cabbage looper *Trichoplusia ni* (Wang et al., 2004), and corn earworm *Helicoverpa zea* (Bayes et al., 2005). In the current research, we report the isolation and characterization of nine unique cDNAs that encode carboxypeptidases from the Hessian fly, *Mayetiola destructor* (Say), one of the most destructive pests of wheat (*Triticum aestivum* L.) (Hatchett et al., 1987; Buntin, 1999). We also analyzed the activity of different carboxypeptidases in different stages and tissues of this insect.

2. Materials and methods

2.1. Insects

The biotype of *M. destructors* used in this research was a Kansas population collected from Ellis County and maintained in the laboratory as described previously (Zhu et al., 2005).

2.2. cDNA library construction and sequencing

The gut was isolated from 200 of 3-day-old *M. destructor* larvae (first instar) by dissection under a microscope and immediately transferred into TRI reagent™ (Molecular Research Center, Inc., Cincinnati, OH, USA). Total RNA was extracted according to the procedure provided by the TRI reagent™ manufacturer. A cDNA library was constructed using the 'SMART™, cDNA library construction kit from Clontech (Palo Alto, CA, USA) according to the manufacturer's instruction with a slight modification. Instead of using the original phage vector, PCR fragments were cloned directly into plasmid pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Recombinant plasmid DNA was isolated with a Qiagen BioRobot-3000 and sequenced using an ABI 3730 DNA analyzer.

2.3. Sequence analysis

Open-reading-frame (ORF) and sequence-similarity analysis were performed using the web-based program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/, Bethesda, MD, USA). Molecular weight calculation and pI prediction of mature proteins were carried out using SDSC biology Workbench (http://workbench.sdsc.edu/). Signal peptides were predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple-sequence alignments and phylogenetic analysis were conducted using BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and Molecular Evolutional Genetics Analysis software (version 2.1).

2.4. RNA isolation and northern blot analysis

Total RNA was extracted from whole insects as above. For Northern blot, 5 μg of total RNA was separated on a 1.2% agarose gel containing formaldehyde and blotted onto a GeneScreen membrane (Perkin-Elmer Life Science Inc., Boston, MA, USA). Membranes were baked at 80 °C for 2 h to fix the RNAs and then hybridized separately to individual cDNAs. Probes were derived from full length cDNAs by labeling with ^{32}P-dCTP using the random labeling kit from Stratagene (La Jolla, CA, USA). Hybridization was performed as described by Chen et al. (2004). After hybridization, the membranes were washed twice with 2 × SSC at room temperature for 30 min, twice with 2 × SSC plus 1% SDS at 65 °C for 30 min, and twice with 0.1 × SSC plus 0.1% SDS at room temperature for 30 min. The membranes were then exposed to Kodak SR-5 X-ray film overnight.

2.5. Carboxypeptidase activity assay

Carboxypeptidase activities were assayed as previously described (Bown et al., 1998; Wang et al., 2004). To determine carboxypeptidase activity in different developmental stages, 0-, 2-, 4-, 6-, and 12-day-old larvae, pupae, and adults were weighted and 20 mg of insects were homogenized in 1 ml distilled water. The protein extract was collected by centrifugation at 13,000*g* for 10 min. One hundred microliters of the protein extract from each developmental stage was mixed with 400 μl of 0.1 M Borate–NaOH buffer (pH 8.0), and the mixture was incubated at 30 °C for 20 min before substrate was added. About 5 μl of 20 mM *N*-(3-[2-furyl] acryloyl)-Phe-Phe (FAPP) (Sigma, St. Louis, MO, USA) was used as the

substrate for carboxypeptidase A while the same amount of *N*-(3-[2-furyl] acryloyl)-Ala-Lys (FAAK) (Sigma) was used as the substrate for carboxypeptidase B. Immediately after the addition of the substrate, optical density on absorbance for the mixture was recorded at 330 nm for 10 min. The rate of hydrolysis of the substrate was calculated as the decrease of the OD_{330} per minute in the linear portion of the initial velocity (Wang et al., 2004). One unit of the enzymatic activity was defined as the hydrolysis of 1 μmol of substrate per min and calculations were performed based on $\Delta\varepsilon_{330} = -2300\,M^{-1}\,cm^{-1}$ for FAPP (Darnis et al., 1999) and $\Delta\varepsilon_{330} = -1820\,M^{-1}\,cm^{-1}$ for FAAK (Plummer and Kimmel, 1980).

To determine the carboxypeptidase activities in different tissues, 250 first instar larvae were dissected. The salivary glands, gut, and the remaining tissues were collected, separately, into 50 μl distilled water. Protein extracts were then obtained and carboxypeptidases A and B activities assayed as described previously.

To determine the carboxypeptidase activities of gut contents, mid-guts were dissected from 2-, 4-, 6-, and 12-day old larvae, separately. Gut contents were collected into 50 μl of distilled water by breaking individual guts using a dissecting needle. Total protein concentration of each collection was determined by Bicinchoninic Acid (BCA) assay. The gut contents were diluted as necessary to obtain same amount of proteins for the carboxypeptidase activity assay.

3. Results

3.1. Isolation of carboxypeptidase cDNAS

To isolate cDNAs that encode digestive carboxypeptidases, a global transcriptomic analysis was conducted using RNA isolated from the gut of first instar larvae. From a total of 1014 randomly sequenced cDNAs, 19 clones were found to encode carboxypeptidase-like proteins based on sequence similarity. Of the 19 clones, nine encoded different proteins while the others are redundant clones. Sequence analysis revealed that five of the nine cDNAs encode proteins with amino acid sequence identity over 95% (data not shown). These similar proteins were named MDCP (*M. destructor* carboxypeptidase) -A1a to -A1e according to the structural similarity with well characterized carboxypeptidase A. The other four cDNAs encode very different proteins, with amino acid sequence identity less than 60%. These different proteins were named MDCP-A2, MDCP-B, MDCP-B-like (MDCP-BL), and MDCP-D, again based on structural similarity with well characterized carboxypeptidases from other organisms. Of the 10 redundant clones, four belong to MDCP-A1a, one to MDCP-A1d, three to MDCP-B, and two to MDCP-BL.

The cDNAs are all full length with sizes ranging from 1.3 to 1.9 kb (Table 1). The predicted proteins are of 415–460 amino acids with p*I* values ranging from 5.15 to 6.5. All proteins have a putative secretion signal peptide present in the first 18–35 amino acid residues at their N-termini. The putative proteins were assigned to either carboxypeptidase A, B, BL, or D according to sequence similarity with previously well-characterized enzymes within the database.

3.2. Structure of the deduced proteins

Fig. 1 shows the alignment of members from MDCP-A1a, MDCP-A2, MDCP-B, and MDCP-BL, along with two known enzymes, *Bos taurus* carboxypeptidase A ((BtCPA) and carboxypeptidase B (BtCPB) (Titani et al., 1975; Goo et al., 1995). All of these proteins have an HXXE motif, which is characteristic of the metallo-carboxypeptidase family (Yan et al., 2002). In addition to the HXXE motif, several other amino acids were also conserved, including His69, Arg71, Glu72, Arg127, Arg145, His196, Tyr248 and Glu270 (Titani et al., 1975). Of the conserved residues, His69, Glu72, and His196 (Gln in the MDCP-A1 group) are involved in zinc binding, whereas Arg71, Arg127, and Arg145 along with Tyr248 (His in the MDCP-A1 group) and Glu270 (either Leu or Ile in the MDCP-A1 group) are involved in substrate binding and cleavage (Auld, 1998; Estebanez-Perpina et al., 2001).

Fig. 2 shows the sequence alignment of MDCP-D with a *Drosophila* and a mouse carboxypeptidase D. MDCP-D and the *Drosophila* protein contain a single

Table 1
Characteristics of the respective cDNA and the putative proteins

cDNA name	Accession no.	cDNA size (bp)	Coding region	Amino acids	Mass (kDa)	p*I*	SSP	Type
MDCP-A1a	DQ196068	1345	36–1283	415	47.82	5.22	18	A
MDCP-A1b	DQ196069	1344	34–1281	415	47.89	5.15	18	A
MDCP-A1c	DQ196070	1343	34–1281	415	47.89	5.15	18	A
MDCP-A1d	DQ196071	1341	34–1281	415	47.79	5.29	18	A
MDCP-A1e	DQ196072	1343	34–1281	415	47.85	5.22	18	A
MDCP-A2	DQ196073	1397	75–1313	412	47.57	6.46	18	A
MDCP-BL	DQ196074	1539	55–1437	460	52.64	5.33	18	Unknown
MDCP-B	DQ196075	1655	56–1390	444	50.51	6.50	21	B
MDCP-D	DQ196076	1894	316–1614	432	49.33	5.46	35	D

Acc. no. and SSP represent GenBank Accession number and secretion signal peptide, respectively.

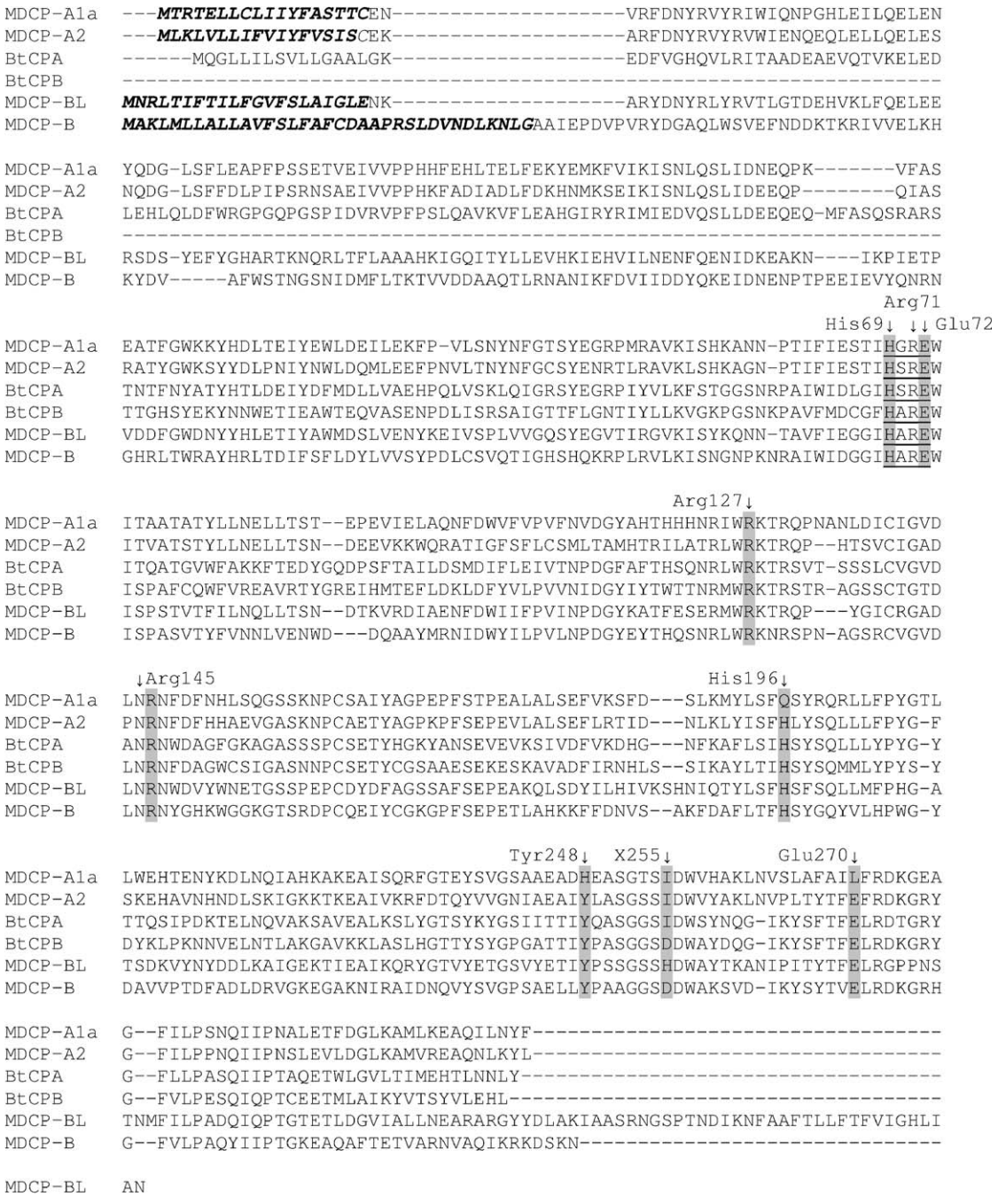

Fig. 1. Amino acid sequence alignment of *M. destructor* carboxypeptidase-like precursors with two known carboxypeptidases. BtCPA and BtCPB are carboxypeptidase A (P00730) and B (P00732) from bovine *Bos taurus*. Putative secretion signal peptides are italic and bold. The catalytic zinc site including His69, Glu72 (HXXE boxes) and His196, and the residues involved in the substrate binding and cleavage activity including Arg71, Arg127, Arg145, Tyr248, X255 and Glu270 are highlighted with arrows in a gray background. The numbers are designed according to bovine carboxypeptidase A (Titani et al., 1975).

carboxpeptidase-like domain with similar size (Sidyelyeva and Fricker, 2002). The sequence identity between MDCP-D and the *Drosophila* carboxypeptidase D is 56%. The mouse protein contains three carboxpeptidase-like domains (Ishikawa et al., 1998). MDCP-D has 45% amino acids identical to domain B of the mouse carboxypeptidase D. The residues characteristic of carboxypeptidase D are all conserved among these three proteins, including zinc binding residues His112, Glu115 and His226, substrate binding residues Arg186, Tyr292, and Gln299, and catalytic residues Gly227, Gly228 and Arg314 (Ishikawa et al., 1998).

3.3. Northern blot analysis

Northern blot analysis with RNA samples isolated from larvae, pupae, and adults were conducted to determine

Fig. 2. Alignment of the *M. destructor* MDCP-D with a *Drosophila* and a mouse carboxypeptidase D. The accession number for the *Drosophila* enzyme is AF545820 and for the mouse carboxypeptidase D is D85391. The putative secretion signal peptide is italic and bold. The zinc binding site His112, Glu115 (HXXE boxes) and His226 (numbers designed according to MDCP-D), the residues involved in substrate binding including Arg186, Tyr292, and Gln299, or involved in catalytic function including Gly227, Gly228 and Arg314, are highlighted with arrows in a gray background.

stage-specific expression using probes specific for each gene. The expression pattern of each gene varied widely in different developmental stages (Fig. 3). Three of the five genes, MDCP-A1, MDCP-A2, and MDCP-B, were exclusively expressed in the larval stage. The expression of MDCP-A1 was low in freshly hatched larvae (0-day-old), but significantly increased in 2-day-old larvae, reaching maximum expression in 4- to 6-day-old larvae before decreasing in 12-day-old larvae. The expression of MDCP-A2 was absent in freshly hatched larvae, low in 2-day-old larvae, high in 4-day-old larvae, low again in 6-day-old larvae, and then high again in 12-day-old larvae. The expression of MDCP-B was high and relatively stable in all larval stages, with maximum expression in 2-day-old larvae.

In contrast, MDCP-BL and MDCP-D were expressed in all developmental stages. The expression of MDCP-BL was low in fresh and 2-day-old larvae, but increased in 4-day-old larvae and reached a maximum in 6-day old larvae, followed by a significant decrease in 12-day-old larvae. Interestingly, the expression of MDCP-BL increased again in pupae and adults, especially in the later stage. The expression pattern of MDCP-D was essentially identical to that of MDCP-B except that the maximum expression occurred in 4-day-old larvae instead of in 2-day-old larvae as observed in MDCP-B, and that a low level of expression was also detected in MDCP-D of pupae and adults.

3.4. Stage-specific enzymatic activity of carboxypeptidases A and B

The activity of carboxypeptidases A and B in different developmental stages was determined using two sets of different samples: whole body extracts (Fig. 4a) and gut content extracts (Fig. 4b). FAPP and FAAK were used as the substrates for carboxypeptidases A and B, respectively. Hydrolytic activities to both substrates were detected in both whole body extracts and gut content extracts. However, the distribution of the activity for carboxypeptidases A and B in these two sets of samples were quite different. In whole body extracts, the activity of carboxypeptidase A was much higher than that of carboxypeptidase B, except in the sample from 12-day-old larvae. The activity of carboxypeptidase A was high in 0- to 6-day-old larvae, with a maximum level in 2-day-old larvae. Conversely, carboxypeptidase A was barely detectable in 12-day-old larvae, pupae, or adults. For carboxypeptidase B, low level activity was detected in all developmental stages, although the activity in samples from 0-, 2- and 4-day-old larvae was slightly higher than that in other

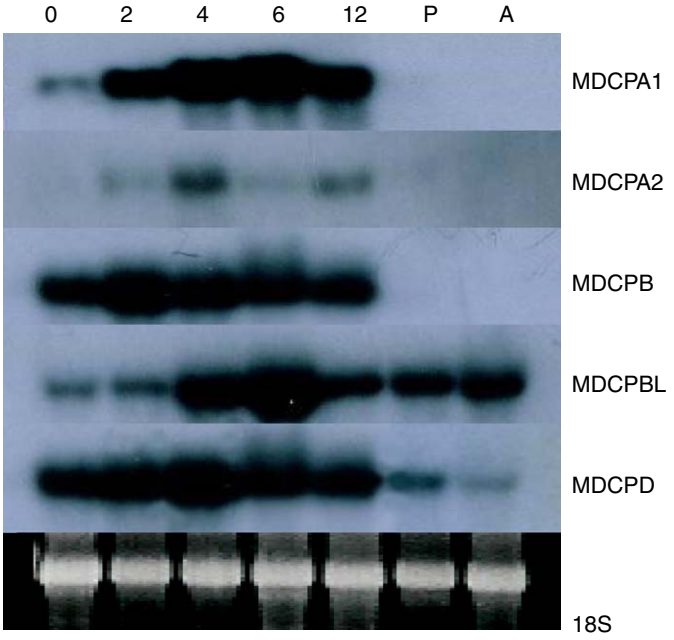

Fig. 3. Northern blot analysis. RNA samples were extracted from 0-day (freshly hatched), 2-, 4-, 6- and 12-day-old larvae, pupae (P), and adults (A), respectively, as indicated in the figure. Probes were derived from cDNAs encoding MDCP-A1a, MDCP-A2, MDCP-B, MDCP-BL, and MDCP-D. Hybridization and washing were performed as described in the Materials and methods section. The image of the 18S rRNA is shown as evidence for relatively equal loading.

stages. In the gut content extracts, the activity for carboxypeptiase A was also much (4–10 times) higher than that for carboxypeptidase B. However, the distribution of carboxypeptidase A activity was significantly different from that observed in whole body extracts. The maximum activity of carboxypeptidase A was detected in 4- to 6-day old larvae.

3.5. Tissue-specific enzymatic activity of carboxypeptidases A and B

The same procedure as described earlier was used to determine tissue-specific enzymatic activities of carboxypeptidases A and B (Fig. 4c). Carboxypeptidase A was detected in all tissues assayed. However, carboxypeptidase B was detected in guts and other tissues, but not in salivary glands. Even though the highest activity of both carboxypeptidase A and B was found in the gut, the activity of carboxypeptidase A was at least four times higher than that of carboxypeptidase B in comparison. Specifically, the activity of carboxypeptidase A in different tissues was 0.0017 ± 0.0006 units per 1000 pairs of glands, 0.0166 ± 0.0033 units per 1000 guts, and 0.0012 ± 0.0002 units per 1000 sets of other tissues. In contrast, the activity of carboxypeptidase B in different tissues was -0.0002 ± 0.0008 units per 1000 pairs of glands, 0.0040 ± 0.0021 units per 1000 guts, and 0.0007 ± 0.0029 units per 1000 sets of other tissues.

4. Discussion

In this study, we cloned and characterized nine cDNAs that encode different carboxypeptidases from the guts of *M. destructor* first instar larvae. Five of the cDNAs encode similar proteins with over 96% amino acid sequence identity. These five cDNAs could represent different alleles of the same gene since the cDNA library was made from 200 guts of *M. destructor* larvae. Alternatively, they could represent different genes that were recently duplicated. The other four cDNAs encode proteins that are very different, with only 60% or less amino acid sequence identity. Apparently, these four cDNAs represent four different genes.

In comparison with well characterized carboxypeptidases from other organisms, the cDNAs encode proteins that belong to three categories of carboxypeptidases, namely: A, B, or D (Titani et al., 1975; Rees et al., 1983; Coll et al., 1991; Guasch et al., 1992; Aloy et al., 1998). In each category, the motifs that are characteristic of carboxypeptidases and residues that are critical for substrate binding were conserved. For example, the motif HXXE (His69 and Glu72 in Fig. 1, His112 and Glu115 in Fig. 2) characteristic of metallocarboxypeptidases was found in all groups. The residues involved in substrate binding and cleavage activity including Arg71, Arg127, and Arg145 were found in both carboxypeptidase A and B (Fig. 1). Similarly, the residues involved in substrate binding including Arg186, Tyr292, and Gln299 and the residues involved in catalytic function including Gly227, Gly228, and Arg314 are all conserved in carboxypeptidase D (Fig. 2). There were, however, a few amino acid residue changes that are known to be important for either enzymatic activity or for substrate specificity in their mammalian counterparts. For example, Tyr248 and Glu270 involved in enzymatic activity in mammalian enzymes were replaced by His248 and Leu270 or Ile270, respectively, in some members of the MDCP-A1 group. Members with one of these two mutations are unlikely to encode catalytically active enzymes since Tyr248 and Glu270 are essential for catalysis. Therefore, the proteins encoded by these genes shall be referred to as carboxypeptidase A-homologues. Fifty-seven genes encoding serine protease-homologues, which are unlikely active due to mutations in key residues, were identified in the *Drosophila melanogaster* genome (Ross et al., 2003). The biological significance of these inactive protease-homologues remains to be resolved. Another example was the residue at position 255, which was critical to determine substrate specificity of the enzyme (Titani et al., 1975). In the carboxypeptidases from mammalian systems, the residue at position 255 is an uncharged residue (Ile255) for carboxypeptidase A and a negatively charged residue (Asp255) for carboxypeptidase B (Titani et al., 1975). In the *M. destructor*, the uncharged Ile255 was conserved in all putative carboxypeptidases A and the negatively charged Asp255 was also found in carboxypeptidase

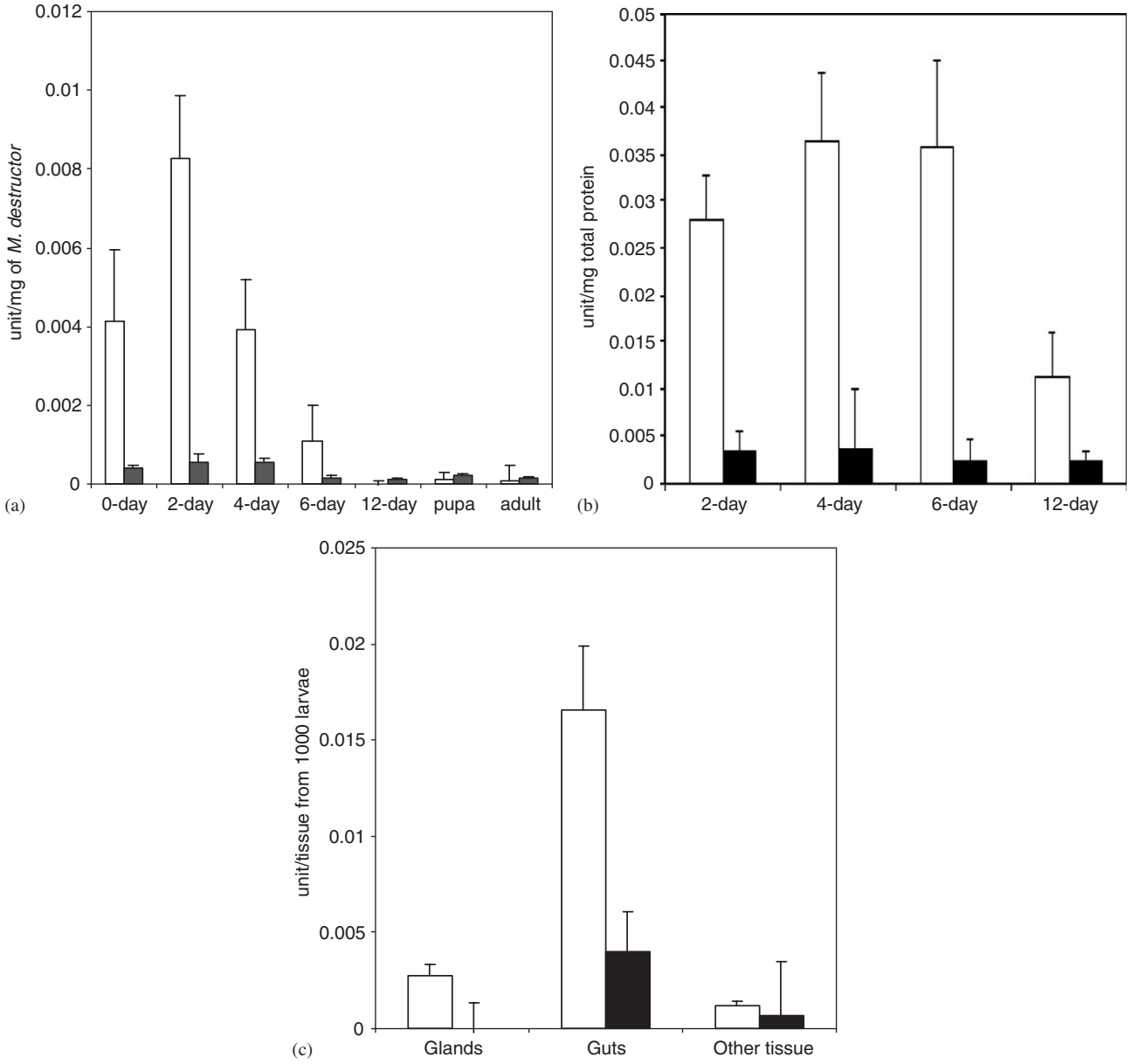

Fig. 4. Activity assays of carboxypeptidases: (a) protease activity in protein extracts derived from whole bodies of different developmental stages. Carboxypeptidase A activity (white) was determined using the substrate FAPP, while carboxypeptidase B activity (black) was determined using the substrate FAAK. Means and standard deviations were derived from three replications. (b) Protease activity in protein extracts derived from gut contents in different stage. The enzymatic activities of carboxypeptidase A (white) and carboxypeptidase B (black) were determined under the same conditions as described in panel A. (c) Protease activity in protein extracts derived from different tissues of first instar larvae. The enzymatic activities of carboxypeptidase A (white) and carboxypeptidase B (black) were determined as described above.

MDCP-B. However, a positively charged residue (His255) was found in MDCP-BL. In mammalian systems, carboxypeptidase A has a preferred specificity for hydrophobic, aromatic and aliphatic amino acid residues, whereas carboxypeptidase B is preferentially specific to basic amino acid residues (Rawlings, 1998). A positively charged residue at position 255 makes MDCP-BL impossible to have substrate specificity towards basic amino acid residues (Bown and Gatehouse, 2004). The exact impact of these amino acid changes on enzymatic activity and/or substrate specificity will remain unknown until further experiments are completed. Amino acid differences in key residues between mammalian enzymes and their insect counterparts have also been reported in other insects previously. For example, a positively charged residue at position 255 was found in a carboxypeptidase B of *G. morsitans* (Lys255) (Yan et al., 2002), and in a carboxypeptidase B of *T. ni* (Arg255) (Wang et al., 2004).

In addition to sequence diversity, expression patterns varied widely among different genes of *M. destructor* carboxypeptidases as demonstrated by northern blot analysis (Fig. 3). MDCP-A1 and MDCP-B were abundantly and exclusively expressed in the larval stages

(Fig. 3), suggesting a role of these carboxypeptidases in food digestion, because the larval stage is the only feeding stage for *M. destructor*. MDCP-A2 was also exclusively expressed in the larval stage. However, MDCP-A2 expression pattern was uneven during larval development. The increased level of MDCP-A2 transcript specifically in 4- and 12-day-old larvae coincided with the period of larval transformation from first to second instar and from second to third instar, respectively, indicating that MDCP-A2 may play a role in the regulation of morphogenesis. MDCP-BL and MDCP-D were expressed in all stages including larvae, pupae, and adults. Apparently, these two groups of enzymes are involved in other proteolyses in addition to their potential role in food digestion.

Enzymatic activities of carboxypeptidase A and B were detected in a few insect species and varied depending on different species (Valaitis et al., 1999; Bown et al., 1998; Wang et al., 2004). Using substrates specific to carboxypeptidase A or B, our results demonstrate that both proteases are predominant in *M. destructor* larvae (Fig. 4). Over 80% of the enzymatic activity for either carboxypeptidase A or B was found in the gut. This observation again strongly suggests that the majority of carboxypeptidase A and B in *M. destructor* are digestive enzymes. It is interesting to note that the distribution of enzymatic activity for carboxypeptidase A was quite different in the whole body extracts and the gut content extracts. In the whole body extract, the highest activity for carboxypeptidase A was detected in the first instar larvae, with a peak in 2-day-old larvae. In comparison, the highest activity for carboxypeptiase A in the gut content extracts was found in 4- to 6-day-old larvae. This discrepancy indicated that there may be other carboxypeptidase A genes that perform functions in other tissues of early larvae. The similarity between carboxypeptidase A activity in the gut content extracts and the expression pattern of carboxypeptidase A1 (MDCP-A1, Fig. 3) indicated that carboxypeptidase A1 is the major digestive enzyme in this insect.

In larval stage, the average activity of carboxypeptidase A from *M. destructor* was ~0.00347 unit/mg of larval protein extract (Fig. 4), which was comparable to 0.00318 unit/mg of midgut extract for the carboxypeptidase A activity from *T. ni* (Wang et al., 2004), but less than 0.00693 unit/mg of gut protein extract for the carboxypeptidase A activity from the western spruce budworm *Choristoneura occidentalis* (Valaitis et al., 1999). The activity of carboxypeptidase A in the gut of *M. destructor* was at least four times higher than carboxypeptidase B (Fig. 4). Similar results were also found between these two types of enzymes in another insect (Wang et al., 2004). This observation suggests that carboxypeptidase A is the major digestive enzyme in insect guts. Alternatively, lower activity for carboxypeptidase B could be due to an imperfect interaction between the enzyme and the artificial substrate, which was designed originally for mammalian enzyme assays.

In summary, we have isolated and characterized cDNA clones that encode different carboxypeptidases from *M. destructor*. Results of northern blot analysis and direct enzymatic assays revealed that carboxypeptidase A1 and B are major digestive enzymes in the gut of *M. destructor* larvae. The other carboxypeptidases including A2, BL, and D are likely to play regulatory roles for insect development in addition to a potential role in food digestion. This research together with previous work on digestive trypsins and chymotrypsins (Zhu et al., 2005) will provide a foundation for future research on the digestive physiology of *M. destructor* and for identification of *M. destructor* protease inhibitors.

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